Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species

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Sex pheromone communication, acting as a prezygotic barrier to mating, is believed to have contributed to the speciation of moths and butterflies in the order Lepidoptera. Five decades after the discovery of the first moth sex pheromone, little is known about the molecular mechanisms that underlie the evolution of pheromone communication between closely related species. Although Asian and European corn borers (ACB and ECB) can be interbred in the laboratory, they are behaviorally isolated from mating naturally by their responses to subtly different sex pheromone isomers, (E)-12- and (Z)-12-tetradecenyl acetate and (E)-11- and (Z)-11-tetradecenyl acetate (ACB: E12, Z12; ECB; E11, Z11). Male moth olfactory systems respond specifically to the pheromone blend produced by their conspecific females. In vitro, ECB(Z) odorant receptor 3 (OR3), a sex pheromone receptor expressed in male antennae, responds strongly to E11 but also generally to the Z11, E12, and Z12 pheromones. In contrast, we show that ACB OR3, a gene that has been subjected to positive selection (ω = 2.9), responds preferentially to the ACB E12 and Z12 pheromones. In *Ostrinia* species the amino acid residue corresponding to position 148 in transmembrane domain 3 of OR3 is alanine (A), except for ACB OR3 that has a threonine (T) in this position. Mutation of this residue from A to T alters the pheromone recognition pattern by selectively reducing the E11 response ∼14-fold. These results suggest that discrete mutations that narrow the specificity of more broadly responsive sex pheromone receptors may provide a mechanism that contributes to speciation.

nubilalis | furnacalis

Sex pheromone communication in the Lepidoptera (moths and butterflies) has fascinated scientists of different disciplines for more than a century (1). Ninety-eight percent of the extant species form a relatively recent monophyletic lineage termed the Ditrysia (2) that is characterized by the use of long-distance sex pheromone communication to mediate mating behavior. Female moths synthesize and emit blends of long chain fatty acid derivatives from a pheromone gland at the tip of their abdomen. This "calling behavior" attracts male moths from distances of up to 100 m (3). In many cases, closely related species can only be differentiated on the basis of subtle changes to the chemical structure and/or ratios of their sex pheromone blends (3, 4). Male moths typically respond with high specificity only to the sex pheromone blend produced by conspecific females. Of several potential barriers leading to reproductive isolation, mate-selection behavior by moths is one of the strongest (5). The specificity of this communication channel is thought to have contributed to the estimated 500,000 species of Lepidoptera (2, 3).

Although the first moth sex pheromone, bombykol, was discovered 5 decades ago (6), little is known about the molecular mechanisms that underlie the evolution of new sex pheromone blends between closely related species. Female sex pheromone production and male detection and response are under the control of different genes (7, 8). Recent studies have identified genetic loci associated with pheromone production and detection that account for the segregation of closely related moth species or races (9, 10). A lineage of conserved odorant receptor (OR) genes expressed in male moth antennae, represent the primary genes responsible for detecting and discriminating female-produced sex pheromones (11–14). The response profile of the sex pheromone receptors (15), or their expression pattern (16), can directly account for the attractive behavioral response of transgenic male silk moths. The specificity of male response, conferred by the sex pheromone receptors, is believed to be subject to strong stabilizing selection that reduces variation in favor of mate selection efficiency (3). The molecular mechanisms that enable male moths to respond to new female pheromones during the evolution of the sexual communication channel remains a long-standing question.

In the moth genus *Ostrinia*, sex pheromones have been identified from eight species (17). The Asian corn borer (ACB, *Ostrinia furnacalis*) and the European corn borer (ECB, *Ostrinia nubilalis*), are part of a closely related lineage termed group III, which also includes *Ostrinia scapulalis*, *Ostrinia zaguliaevi*, and *Ostrinia zealis*. Three other species, *Ostrinia latipennis*, *Ostrinia ovalipennis*, and *Ostrinia palustralis*, constitute a more distantly related lineage termed group II (17–19) (Fig. S1). Collectively these species use (E) and (Z) isomers of tetradecenyl acetate and tetradecenol in distinct combinations to define their pheromone specificity. With the exception of ACB, group III species use varying ratios of (E)-11- and (Z)-11-tetradecenyl acetate (E11 and Z11) in their sex pheromone blend (17). Further, two races of ECB produce and respond to opposite ratios of the E11 and Z11 pheromones, ECB (E) and ECB(Z), respectively (20). ACB is unique within the *Ostrinia*, having evolved to use an acetate pheromone with a shift in the location of the double bond, (E)-12- and (Z)-12-tetradecenyl acetate (E12 and Z12) (Fig. S1) (21, 22). ACB and ECB provide an unprecedented opportunity to elucidate molecular mechanisms underlying the shift in specificity of the male moth in response to a new pheromone structure produced by the female.

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Five different sex pheromone receptors have recently been identified from ECB(Z) and *O. scapulalis*. A few of these receptors have been functionally characterized in vitro using *Xenopus* oocytes (13, 14), a system that is well established for recording responses of insect ORs (12). Some of these receptors responded broadly to sex pheromone components in general (13, 14), but others like ECB(Z) OR6 were found to respond almost exclusively to Z11, the primary pheromone produced by ECB(Z) females (14). We cloned the orthologous receptors from ACB and analyzed their sequences, finding no evidence for changes in gene expression levels between the two closely related species. However, tests of molecular evolution of the protein-coding sequences suggested that the ACB OR3 lineage had been uniquely subjected to positive selective pressure. Responses in *Xenopus* oocytes confirmed an evolved function of ACB OR3 that exhibited an increased selectivity to E12 and Z12 pheromones compared with its ECB(Z) OR3 ortholog. A single amino acid polymorphism at position 148 of the predicted third transmembrane domain (TMD3) controlled the selective response. The data suggest that discrete mutations in OR genes can result in cladogenic changes in pheromone recognition patterns.

Results

Sixteen pheromone receptors, orthologs of ECB(Z) ORs 1 and 3–6 (14), were cloned from the male antennae of ACB, ECB(E), and ECB(Z) (GenBank accession nos. JN169130–42). Insects reared at the New York State Agricultural Experiment Station (NYSAES) were used because they have served as a historical genetic stock for experiments comparing the different species and races (23–25). To avoid biasing the coding region sequence, each ORF was cloned using primers designed to recognize the 3′ and 5′ UTR of the OR sequences (14).

No Evidence for Changes in OR Gene Expression Between ACB and ECB. ACB and ECB(Z) antennae were assayed by quantitative PCR (qPCR) for changes in *OR* gene regulation that might be associated with new function. Consistent with its functional role as an obligate dimer partner in the neuron membrane, *OR* coreceptor (Orco) was expressed as highly as the reference gene, *RpS3*, and without significant sexual bias (Fig. S2). *ORs* 1 and 3–6 were all expressed at significantly higher levels in male antennae, consistent with their role in detecting female-produced sex pheromone (Fig. S2). On average, the expression levels of *ORs* 1 and 3–6 were at least 106-, 545-, 75-, 23-, and 362-fold higher in male compared with female corn borer antennae, respectively. Notably, no significant differences in *OR* gene expression levels were detected between ACB and ECB(Z).

Evidence for Positive Selection Acting on ACB OR3. In addition to the 16 sequences reported here, 38 orthologous sequences (13) representing eight different *Ostrinia* species were downloaded from the National Center for Biotechnology Information GenBank and used to construct a neighbor-joining phylogenetic tree (Fig. 1). In general the receptor nomenclature reported in ref. 14 is used, except in Fig. 1, where receptors reported in ref. 13 retain their original name as published. These 54 sequences form five orthologous lineages, each with 100% bootstrap support, and all belong to the Lepidoptera pheromone receptor lineage. OR1 sequences form two separate lineages with 92% bootstrap support (Fig. 1). The two OR1 lineages share 83% nucleotide identity, but without knowledge of their gene synteny, it is difficult to determine whether they represent different genes or alleles; therefore, OR1 was treated as a single lineage for analyses of sequence evolution.

To investigate evolutionary pressures acting on the coding regions of *Ostrinia* sex pheromone receptor genes, we estimated the rates of synonymous (dS) and nonsynonymous (dN) nucleotide substitutions in the five gene lineages using branch-specific

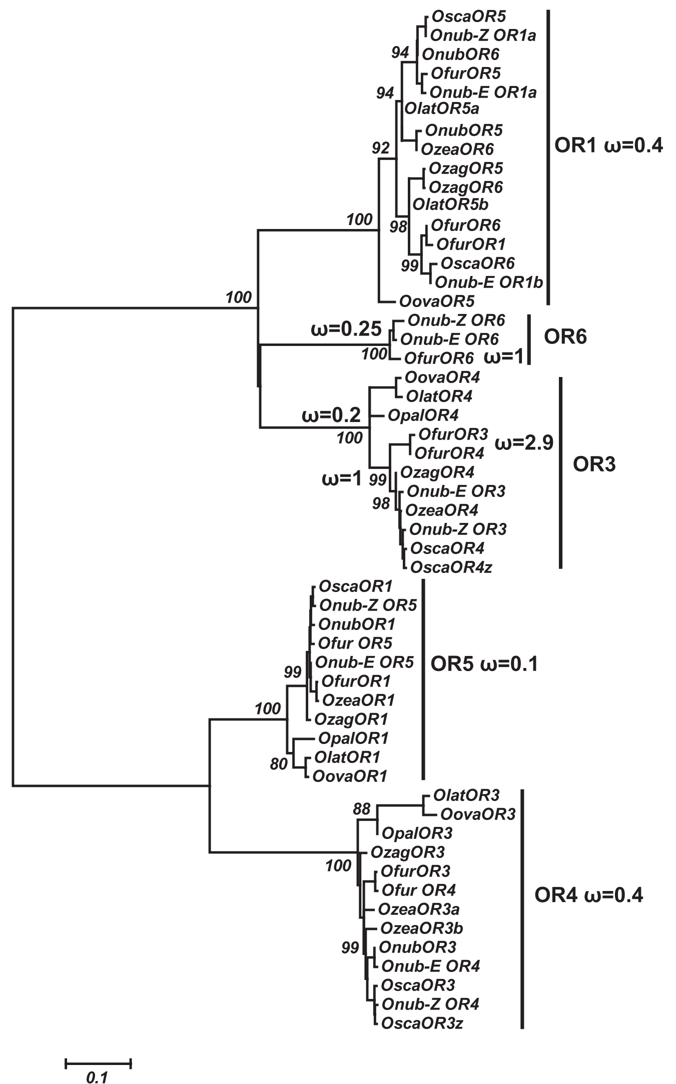

Fig. 1. Evidence of positive selection acting on the coding sequence of ACB OR3. A total of 54 nucleotide sequences representing eight species and five OR gene lineages were analyzed for evidence of selection. Bootstrap values for major branches are shown as a percentage of n = 1,000 replications. The normalized nonsynonymous to synonymous substitution rate (ω) is shown for significant groupings. ORs 1, 4, and 5 have one uniform ω for all branches in the gene lineage, whereas ORs 3 and 6 have two or more rates (ω) for branches within the gene lineage. ω > 1 was observed only for ACB OR3, suggesting that positive selection has acted on this branch. OR nomenclature follows their original publication (13, 14). *O. furnacalis* (ACB), *O. latipennis*, *O. ovalipennis*, *O. nubilalis* [ECB(E)] and [ECB(Z)], *O. palustralis*, *O. scapulalis*, *O. zaguliaevi*, and *O. zealis*.

models (26, 27). For OR lineages 1, 4, and 5 the one ratio model (M0) was not rejected (Table S1). The normalized d_N/d_S ratio (ω) for each of these lineages was significantly less than 1 (Fig. 1), consistent with purifying selection. The free ratio model (M1) was a significantly better fit than M0 for OR lineages 3 and 6, suggesting that selective pressure varies among different branches within these lineages (Table S1). Although the number of sequences in the OR6 lineage is small, the branch leading to ACB OR6 seems to have been released from purifying selection (ω = 1; Fig. 1), and may reflect the lack of Z11 pheromone production by ACB females. The OR3 gene lineage produced the most interesting results. The branch leading to the three ancestral group II species (*O. latipennis*, *O. ovalipennis*, and *O. palustralis*) exhibited strong evidence for purifying selection (ω = 0.2, $P < 0.001$).

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With the exception of ACB, the branch leading to all group III species seems to have been subject to neutral or relaxed purifying selection. Only the branch leading to ACB OR3, and not the branch leading to ECB(E) and ECB(Z), yielded a ω value greater than 1, providing evidence for positive selection acting on ACB OR3 (Fig. 1; $\omega = 2.9$, $P = 0.008$).

OR3 from ACB Displays Greater Response Specificity to E12 and Z12 Pheromones. With evidence of positive selection acting on ACB OR3, we compared its functional response in vitro with its ECB (Z) ortholog. Previously, insect ORs have been characterized as ligand-gated nonselective cation channels, although the mechanisms of channel activation and function remain unresolved (28, 29). Insect ORs are unique in that they all require Orco as a chaperone and coreceptor to functionally express both in vivo as well as in isolated expression systems (12, 30). Here, we use the *Xenopus* oocyte system to assay the response of OR3 from ACB and ECB(Z) to the four group III pheromones with isomers at position 11 and 12 (E11, Z11, E12, and Z12). Each receptor was coexpressed with the obligate Orco partner from ECB(Z) (GenBank accession no. ADB89179); ECB(Z) and ACB Orco (GenBank accession no. BAJ23261) have identical amino acid sequences. Pheromone application to voltage-clamped oocytes expressing the receptor pairs caused an inward current that was concentration-dependent and saturable. E11 or E12 (1 μM) was applied at the end of each experiment for ECB(Z) and ACB OR3, respectively, to normalize the response of each pheromone (Fig. 2 *A* and *B*).

A concentration–response analysis of each pheromone showed that ECB(Z) OR3 responded to all four pheromones but had the highest affinity and largest response for E11 ($EC_{50} = 12.5$ nM; Fig. 2*C* and Table 1), a response pattern very similar to its ortholog from the closely related species *O. scapulalis* (13). In contrast, ACB OR3 responded with the highest affinity and efficacy to E12 and Z12 pheromones ($EC_{50} = 7.0$ and 9.6 nM, respectively; Fig. 2*D* and Table 1), suggesting it has evolved specificity for the ACB female mating pheromone that contains equal amounts of E12 and Z12 (Fig. S1). The 14-fold lower affinity of ACB OR3 for E11 ($EC_{50} = 179$ nM; Table 1) compared with ECB(Z) OR3 was the most notable difference.

Single Amino Acid Polymorphism Provides a Significant Adaptive Mutation. We conducted mutagenesis experiments to determine residue(s) responsible for the shift in pheromone specificity (Fig. 3). ACB OR3 differs from ECB(Z) OR3 at 47 amino acid positions, and all but three are located in the first half of the protein. We successively mutated 27 of these positions on the basis of their location within predicted transmembrane and extracellular domains expected to interact with the pheromone ligand, to the corresponding ECB(Z) OR3 residue (Fig. S3). Using this approach we identified mutation T148A within the predicted third TMD of ACB OR3 (Fig. 4*A*) as affecting the receptor's affinity for E11, increasing it ∼12-fold (Fig. 3 and Table 1). ACB OR3 has a threonine at position 148, whereas all other orthologs known from *Ostinia* species, including ECB, have an alanine (Fig. 4*B*). The response profile of the converse mutation in ECB(Z) OR3 (A148T) looked strikingly similar to wild-type ACB OR3, with an increased selectivity for E12 and Z12 (Fig. 3). Overall, the concentration–response profiles of the complementary mutants indicate that the unique identity of residue 148 as Thr or Ala accounts for the receptor's response to E11. Mutation of Thr-148 to Ala shifts ACB OR3's EC_{50} for E11 from 179 to 15.3 nM, resembling the wild-type ECB(Z) EC_{50} of 12.5 nM (Fig. 3*B* and Table 1). The EC_{50} of the converse mutation ECB(Z) $OR3^{A148T}$ is shifted oppositely, to 110 nM from the wild-type 12.5 nM. Mutations ECB (Z) $OR3^{A148T}$ and ACB $OR3^{T148A}$ had no statistically significant effect on the affinities or relative efficiencies observed for Z11, E12, or Z12 (Table 1 and Fig. S4). However, ECB(Z) OR3 had

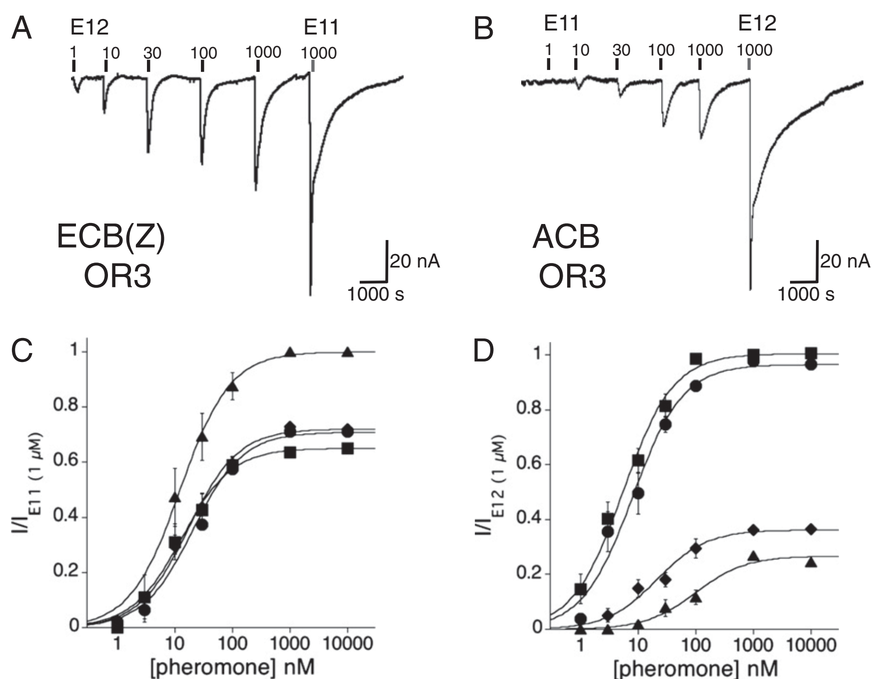

Fig. 2. ACB OR3 responds preferentially to the (E)-12- and (Z)-12-tetradecenyl acetate pheromones produced by ACB females. (*A* and *B*) Representative traces show membrane currents in *Xenopus* oocytes coexpressing OR3 receptors from ECB(Z) (*A*) and ACB (*B*) with the obligate partner Orco. A saturating dose of E11 or E12 (1 μM) was applied at the end of each experiment for ECB(Z) OR3 or for ACB OR3, respectively, to normalize the response of each pheromone. (*C* and *D*) Concentration-dependence of ECB(Z) OR3 (*C*) and ACB OR3 (*D*) responses to E11 (diamonds), Z11 (triangles), Z12 (circles), and E12 (squares) pheromones. The responses were normalized to saturating (1 μM) concentrations of E11 [ECB(Z)] or E12 (ACB) pheromones applied at the end of the experiment.

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Table 1. Affinity and efficiency of ACB and ECB(Z) OR3 responses to four pheromones

	ACBOR3		ECB(Z)OR3		ACBOR3 T148A		ECB(Z)OR3 A148T	
Ligand	EC_{50} (nM ± SEM)	Relative efficiency	EC_{50} (nM ± SEM)	Relative efficiency	EC_{50} (nM ± SEM)	Relative efficiency	EC_{50} (nM ± SEM)	Relative efficiency
E11	179 ± 80 *n* = 5	1	12.5 ± 3.7 *n* = 5	1	15.3 ± 3.1 *n* = 5	1	110 ± 30 *n* = 5	1
Z11	25 ± 7.7 *n* = 5	1.34 ± 0.21	18.7 ± 2.5 *n* = 5	0.72 ± 0.20	6.3 ± 0.75 *n* = 5	1.24 ± 0.26	47.5 ± 4.8 *n* = 5	1.08 ± 0.07
E12	7.0 ± 0.96 *n* = 5	3.75 ± 0.63	13.6 ± 4.7 *n* = 5	0.65 ± 0.11	7.0 ± 1.09 *n* = 5	1.07 ± 0.5	22.9 ± 5.9 *n* = 5	1.75 ± 0.26
Z12	9.6 ± 1.8 *n* = 5	3.61 ± 0.31	20.7 ± 2.4 *n* = 5	0.71 ± 0.06	8.4 ± 1.36 *n* = 5	1.10 ± 0.14	24.0 ± 6.7 *n* = 5	1.60 ± 0.41

The EC_{50} values were estimated by fitting the dose–response data to the simple binding isotherm (I = Imax*[pheromone]/(EC_{50} + [pheromone])). The amplitude measured in response to a saturating (1-μM) dose of the respective pheromones was normalized to that measured for E11. The number of experiments (*n* = 5) performed for wild-type ACB and ECB(Z) OR3, and complementary mutations ECB(Z) $OR3^{A148T}$ and ACB $OR3^{T148A}$, are listed below the EC_{50} value. EC_{50} values were analyzed by two-way ANOVA. Pheromones (*P* = 0.001) and receptors (*P* = 0.006) were significant factors, as was their interaction (*P* = 0.003). The E11 response for ACB OR3 and ECB(Z) $OR3^{A148T}$ were not significantly different from each other but were the only two conditions that were significantly different compared with other receptor and pheromone combinations ($P < 0.05$, Tukey honestly significant difference test).

slightly higher affinity for Z11 than ACB OR3, and T148A increased the affinity of ACB OR3 for Z11, whereas A148T decreased affinity of ECB OR3 for Z11. Consistent with previous topology determinations of insect ORs (30–32), OR3 is predicted to have seven transmembrane domains with an intracellular N terminus and an extracellular C terminus (Fig. 4*A*; TOPCONS, topcons.net). Residue 148 is located within TMD3 near the extracellular surface.

Discussion

In the insect order Lepidoptera, pheromone emission by females and detection by males constitutes a species-specific communication channel that serves as a behavioral prezygotic barrier to mating, enabling closely related species to coexist in the same region (3). A variety of evolutionary mechanisms have been proposed to act on the communication channel, including stabilizing selection, asymmetric tracking, reinforcement, and communication interference (3, 33, 34). How the specificity of the communication channel changes during speciation, including the molecular mechanisms of male detection, has remained a key question (3, 34). We find that a single amino acid polymorphism in ACB OR3 provides a major adaptive mutation that narrows its response specificity to correspond to the E12 and Z12 pheromones produced by the ACB females (21, 22). Interestingly, purifying selection acting on the OR3 lineage of group II species seems to be relaxed in group III species that include the ACB (Fig. 1). Neutral rather than purifying selection acting on the ancestral ACB OR3 gene may have resulted in more nonsynonymous substitutions from which the unique threonine polymorphism was positively selected.

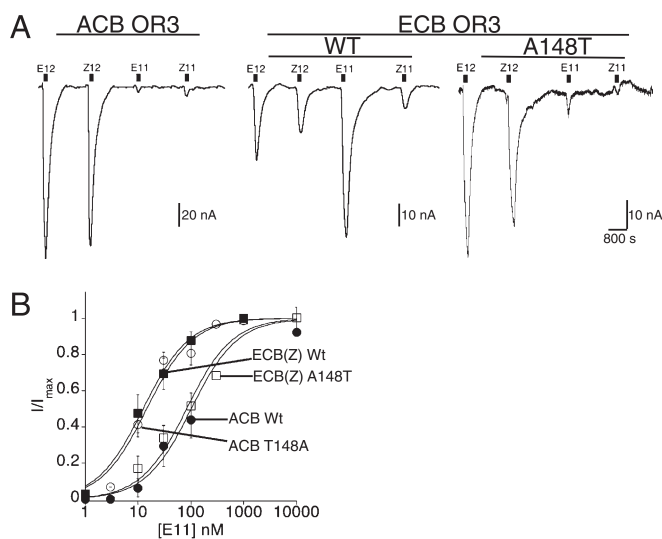

Fig. 3. Identity of amino acid residue 148 of ACB and ECB(Z) OR3 determines differential affinity for E11. (*A*) Representative traces show membrane currents in oocytes coexpressing ACB OR3, ECB(Z) OR3, and ECB(Z) $OR3^{A148T}$ with Orco, in response to 10-nM doses of E11, Z11, E12, and Z12. (*B*) Concentration-dependence of ECB(Z) OR3 (filled squares), ACB OR3 (filled circles), ECB(Z) $OR3^{A148T}$ (open squares), and ACB $OR3^{T148A}$ (open circles) receptors to E11. The responses were normalized to a saturating 1-μM concentration of E11 applied at the end of the experiment.

In many cases the pheromone communication channel of closely related lepidopteran species differs by the ratio of individual components used in the blend, and not by changes in the chemical structure. Down-regulation of the bombykol receptor in the antennae of male silkmoths was recently shown to alter the male response (16). We did not detect any changes in expression of the *Ostrinia* pheromone receptors in ACB and ECB(Z) antennae (Fig. S2), similar to results obtained using closely related *Heliothis* moth species (35). It was concluded that interspecific sequence differences, rather than regulation of gene expression, underlie the species-specific male response of *Heliothis* (35). In support of this hypothesis, introgression of a discrete genomic region encoding four sex pheromone receptors explained the different responses of *Heliothis subflexa* and *Heliothis virescens* males to the pheromone components (9).

Ostrinia species provide an excellent model to elucidate the molecular mechanisms underlying the evolution of male moth olfactory response to new pheromone structures and blends. Although the majority of species studied to date use different ratios of E11 and Z11 as their pheromone, ACB is unique in the genus using an acetate pheromone blend with a shift in the location of the double bond, E12 and Z12 (Fig. S5). This subtle structural change imparts species specificity in the communication channel. Currently, E12 and Z12 are unique pheromones within Lepidoptera [The Pherobase (36)], suggesting that one or more of ACB's receptors have evolved specificity during or after its speciation. We analyzed five pheromone lineages representing eight species (13, 14) for evidence of positive selection. On the basis of normalized nonsynonymous/synonymous nucleotide substitution ratios in the receptor coding region (ω; Fig. 1), OR lineages 1, 4, and 5 exhibited evidence of strong purifying selection (ω = 0.4, 0.4, and 0.1, respectively). OR5 cloned from both group

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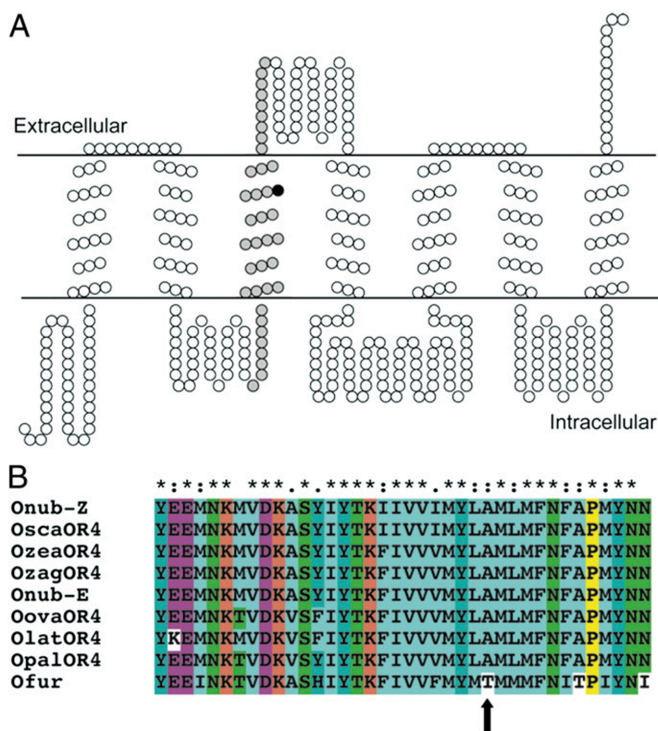

Fig. 4. *Ostrinia* OR3 sequences showing the unique threonine (T) for alanine (A) substitution at position 148 within TMD3 of ACB OR3. (*A*) A TOPCONS (topcons.net) model of ACB OR3 with position 148 illustrated by a black circle. The residues included in the alignment in Fig. 4*B* are highlighted in gray. (*B*) Clustal alignment of the predicted TMD3 of OR3 from eight Ostrinia species. *O. furnacalis* (ACB), *O. latipennis*, *O. ovalipennis*, *O. nubilalis* [ECB(E) and ECB(Z)], *O. palustralis*, *O. scapulalis*, *O. zaguliaevi*, and *O. zealis*.

II and group III species in the *Ostrinia* genus responded specifically to (E)-11–14:OH (37), suggesting that its function has been conserved within the genus. Most functional genes are subject to strong purifying selection that reduces nonsynonymous polymorphisms in these genes (38, 39). Only the branch leading to ACB OR3 showed signs of positive selection, $\omega = 2.9$ (Fig. 1), and it responded in vitro with more specificity to the E12 and Z12 pheromones compared with its ECB(Z) ortholog (Fig. 2).

The unique threonine amino acid at position 148 of ACB OR3 narrows the response spectrum of the receptor by reducing its affinity for E11 by 14-fold compared with ECB(Z) OR3 (Fig. 3 and Table 1). Mutating this position back to alanine (ACB $OR3^{T148A}$) restores sensitivity to E11. Further, the sensitivity of ECB(Z) OR3 to E11 can be reduced by mutating alanine 148 to threonine (Fig. 3*B* and Table 1). Thus, this single mutation in predicted TMD3 of ACB OR3 (Fig. 4) significantly narrows its pheromone selectivity pattern. Smaller changes in affinity to other pheromones were noted, and although other polymorphic domains in the receptor may underlie these slight differences, they were not statistically significant (Table 1).

Often the response spectra of the OR and the olfactory neuron that express it are the same (40–42). *O. scapulalis* OR3 (BAI66606.1), 99% identical to ECB(Z) OR3 in this study, is expressed in pheromone-sensitive olfactory neurons (13). The in vitro response of ACB, ECB(E), and ECB(Z) OR3 to the four pheromones is almost identical to the response profile of pheromone-sensitive olfactory neurons on male antennae, characterized using single sensillum electrophysiology (43, 44). Each sensillum typically houses three different olfactory neurons that have been identified by their spike amplitude: small, intermediate, or large. The response spectra of ECB(E) and (Z) OR3 is similar (E11 > E12 > Z11 > Z12) and corresponds closely to that of the small spiking neuron of ECB(Z) and the large spiking neuron of ECB(E). These results suggest a race-associated change in the location of their expression. The large spiking neuron of ACB responds best to E12 and Z12, whereas its small spiking neuron responds specifically to E12. These results suggests that the ACB large spiking neuron phenotype could have resulted from a T148A mutation of an ancestral ECB(E) OR3 allele because both are associated with the large spiking neuron. The existence of an ACB small spiking neuron that responds specifically to E12 suggests that another OR has evolved different specificity in this system. Additional candidate sex pheromone receptor genes have been identified recently, by sequencing ECB genomic DNA (45).

Ostrinia species have been used as a model to study the evolution of sex pheromone communication between closely related species for more than 3 decades. The discovery of a Δ14-desaturase gene uniquely expressed in the pheromone gland of ACB that produces its unique pheromone advanced the understanding of evolutionary differences between ECB and ACB (46). Interestingly, it was also discovered that 3–4% of ECB males can respond to both the ECB and ACB pheromones, suggesting that variability in the breadth of male response required to track new pheromones exists in the population (46, 47). ECB possesses pheromone-sensitive olfactory neurons that can respond to all four pheromones, and altering the tuning profile of this broadly responsive neuron could explain the evolution of the male ACB olfactory response (23). Adding to this body of knowledge, we have identified a single mutation to ACB OR3 that produces a major shift for specificity to the new ACB pheromone.

Materials and Methods

Insects. Colonies of bivoltine ECB(E) and univoltine ECB(Z), collected from corn fields in western New York state in the late 1990s, are maintained on artificial diet (23–25). For gene expression studies batches of antennae from 1- to 3-d-old adults were collected from ECB(Z) male and female moths (n = 95, 59, and 111 female, and 96, 67, and 120 male moths). The ACB colony originated from the National Institute of Crop Sciences, South Korea, and rearing is the same as described for ECB. ACB antennae dissected at NYSAES, preserved in RNAlater (Ambion), and shipped to Bozeman in three batches of 50–75 moths per batch per sex.

Cloning Full-Length ORs. ECB and ACB antennae were prepared for RNA extraction using previously published methods (48). Full-length ORs were amplified from male ACB, ECB(E), and ECB(Z) antennal cDNA using primers designed to the 3′ and 5′ UTRs. Amplification reactions were performed using Phusion High-Fidelity DNA polymerase (Finnzymes), and at least three different clones were sequenced from both directions for each gene (14).

Phylogenetic and Sequence Analysis. PAL2NAL software was used to generate a multiple codon alignment (49, 50) from the MUSCLE amino acid alignment and the corresponding nucleotide sequences of the *Ostrinia* ORs. A phylogenetic tree was created using MEGA 4 based on the multiple codon alignment (51). The neighbor-joining method was used to estimate evolutionary distances in units of base substitutions per site (52). Tests of selection were performed using the codeml procedure implemented in the PAML 4.4 package (26) that estimates ratios of the normalized nonsynonymous (d_N) to synonymous (d_S) substitution rate (ω) by the maximum likelihood method (53) (*SI Materials and Methods*).

Quantitative Real-Time PCR. qPCR was performed as described previously (48). ORs 1, 3, 4, and 5 primer sets spanned an intron, and the absence of PCR products resulting from contaminating genomic DNA was confirmed by gel electrophoresis and melting temperature analysis. Expression levels less than 10^{-3} of RpS3 were conservatively estimated at 0.001 for calculating fold differences between male and female antennae.

Receptor Functional Analysis. ORs were cloned into the expression vector pGEMHE and analyzed using the *Xenopus* ooctye assay (14) (*SI Materials and Methods*). Individual point mutations were incorporated into the cDNA using the QuikChange Site Directed Mutagensis Kit (Agilent). Data were analyzed offline with Axograph X (v1.3.1) and Kaleidagraph (v4.04) software.

EVOLUTION

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